SHORT RESEARCH ARTICLE

REVISED Long term delivery of pulsed magnetic fields does not alter visual discrimination learning or dendritic spine density in the mouse CA1 pyramidal or dentate gyrus neurons [v2; ref status: indexed, http://f1000r.es/2gk]

Previously titled: Long term delivery of pulsed magnetic fields does not improve learning or alter dendritic spine density in the mouse hippocampus

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Abstract

Repetitive transcranial magnetic stimulation (rTMS) is thought to facilitate brain plasticity. However, few studies address anatomical changes following rTMS in relation to behaviour. We delivered 5 weeks of daily pulsed rTMS stimulation to adult ephrin-A2$^{-/-}$ and wildtype (C57Bl/6j) mice (n=10 per genotype) undergoing a visual learning task and analysed learning performance, as well as spine density, in the dentate gyrus molecular and CA1 pyramidal cell layers in Golgi-stained brain sections. We found that neither learning behaviour, nor hippocampal spine density was affected by long term rTMS. Our negative results highlight the lack of deleterious side effects in normal subjects and are consistent with previous studies suggesting that rTMS has a bigger effect on abnormal or injured brain substrates than on normal/control structures.

Article Status Summary

Referee Responses

Referees	1	2
v1 published 09 Sep 2013	✓ report	✓ report
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1 **Antoni Valero-Cabre**, Boston University USA

2 **Anthony Hannan**, The Florey Institute of Neuroscience and Mental Health Australia

Latest Comments

No Comments Yet

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REVISED **Changes from Version 1**

We would like to thank both reviewers for their thoughtful and constructive reviews of our manuscript. We believe that they have raised important points and we have tried to address and reflect their concerns in our revised manuscript. In particular we clarify aspects of our experimental design, we address up front the limitations of presenting a triple negative result and we emphasize that our results are relevant primarily to the use of low intensity magnetic fields. We are particularly grateful to both reviewers for the opportunity to develop key ideas in the discussion: these include the necessity for fundamental animal studies that will provide insight into what rTMS does to the brain, and the associated importance of designing clinically relevant animal coils to be able to do these studies properly. The title of the article was changed to reflect the findings of the study more precisely in response to the reviewers comments.

See referee reports

Introduction

Repetitive transcranial stimulation (rTMS) generates electrical currents in the brain by electromagnetic induction and has been shown to induce synaptic plasticity in human and animal models[1]. Importantly, rTMS induces long term potentiation (LTP) in rodent hippocampus *in vitro*[2] and several sessions of high-frequency rTMS increases the capacity to induce LTP compared to untreated controls, suggesting it may also regulate metaplasticity[3,4]. Because rTMS acts on the same plasticity mechanisms as learning and memory, it has been hypothesised that rTMS may serve as a "priming" mechanism to facilitate long-term synaptic and structural modifications[5,6]. The implication is that repeated rTMS stimulation sets up a "plastic" brain state that is conducive to long term functional and structural changes[5]. For this reason, rTMS is being explored in combination with behavioural training tasks to see whether it can be used to prime or improve learning and cognitive performance in humans[7,8]. However, the potential mechanisms whereby rTMS might accelerate learning remain unknown.

Declarative and spatial learning tasks are strongly associated with the hippocampus. More specifically, hippocampal dendritic spines have been identified as the likely loci of activity-dependent synaptic plasticity and possible structural correlates of memory and learning[9,10]. High dendritic spine density in hippocampal neurons is associated with learning ability and higher performance on cognitive tasks[11–14]. Furthermore, LTP and LTD induce structural changes in dendritic spines, with LTP induced electrically or via learning increasing dendritic length and spine density[15,16]. Because higher spine density is associated with higher spine mobility and turnover rates[15,16], this measure is thought to reflect a greater capacity for synaptic reorganisation.

To date, the only study to examine changes in dendritic spines after rTMS did so following a single stimulation and showed no change in spine density, although the size of the smallest spines was increased[2]. Therefore, very little has been done to investigate the impact of long term rTMS on spine density in the hippocampus, or how it might interact with the learning process. Given the significant structural changes induced in the mouse visual system following repeated stimulation sessions, and evidence for structural changes in the human brain[17], we hypothesised that a similar long-term rTMS regime in combination with a hippocampus-dependent learning task, would rescue impaired learning strategies previously found in ephrin-A2$^{-/-}$ mice[18] and alter spine density in the hippocampus.

We delivered 5 weeks of daily pulsed rTMS stimulation to ephrin-A2$^{-/-}$ and wildtype mice undergoing a visual learning task and analysed learning performance, as well as spine density in the dentate gyrus molecular and CA1 pyramidal cell layers in Golgi-stained material.

We used ephrin-A2$^{-/-}$ mice because they have previously been shown to have a specific learning deficit[18]. In addition, although ephrin-A2 is expressed in the mouse hippocampus throughout life and has been implicated in its topographic organisation[19,20], there is no evidence that it is involved in synaptic plasticity or spine dynamics[21]. Thus we aimed to examine a learning-mediated effect of rTMS on dendritic spines. Although mice of both genotypes learned the task, their performance remained suboptimal due to lack of motivation to obtain food rewards through insufficient food restriction[22] and neither learning behaviour, nor hippocampal spine density were affected by long term rTMS. Our negative results are consistent with previous data showing that rTMS has a selective effect on abnormal or injured brain circuitry[23], and the lack of deleterious side effects observed in normal human subjects[8,24].

Methods

Animals

This experiment used 10 wildtype (C57Bl/6J) and 10 ephrin-A2$^{-/-}$ knockout mice, with equal number of males and females. Wildtype mice were purchased from Animal Research Centre (Canning Vale, WA, Australia). Ephrin-A2$^{-/-}$ mice were a generous gift from David Feldheim[25] and carry a homozygous null mutation of the ephrin-A2 gene. Ephrin-A2$^{-/-}$ mice were bred from heterozygous parents at the Biomedical Research Facility (The University of Western Australia) and backcrossed for >10 generations on a C57Bl/6J background. Randomised littermates were not used because the breeding colony was structured to produce ephrin-A2/A5 double knockout mice for other studies and no WT littermates were obtained. Mice were genotyped at weaning, as described previously[25]. Mice were age matched, aged 8–10 weeks old (equivalent to young sexually mature adult in humans) when commencing the experiment. For the duration of the study, mice were kept in standard caging in a controlled environment (12/12 light/dark cycle; temperature 22°C±2°C, separated into cages with clear plastic walls (17 cm × 19 cm base, 16 cm high) based on sex and genotype (2–4 per cage)). Food restriction began two days prior to commencing training. This aimed to reduce mice to 90% of their free-feeding body weight. Mice were weighed daily and food intake adjusted using a daily-based controlled diet to reach and maintain target body weights and ensure animals remained healthy. Water was available *ad libitum* throughout the experiment. All procedures in this study were conducted in accordance with US NIH guidelines and approved by The University of Western Australia Animal Ethics Committee.

Apparatus and procedure

Mice completed a visual discrimination task in two phases. Mice were initially rewarded for one stimulus ('learning phase'). After mice learned the task (defined as 75% correct responses for three consecutive days), the rewarded stimulus was switched to the opposite, previously incorrect stimulus ('reverse phase'). rTMS was applied

(as described below) for 10 minutes daily immediately following the task during the reverse phase. We chose to stimulate after the task because we hypothesized that rTMS would enhance LTP-like processes, stabilizing new spines, and the associated synaptic connections, that had formed during learning. Because most mice in the study failed to learn the reverse task, we decided to focus on the relationship between rTMS and dendritic spine density, therefore, mice were terminally anesthetised with pentobarbitone (Lethabarb, Virbac Australia, 160 mg/kg, i.p.) 24 hours after 35 days of rTMS so that all mice received the same amount of stimulation.

Visual discrimination task

The visual discrimination task was carried out using a Y-maze fitted into a 50 cm^2 box, with visual stimuli at each end of the Y maze arms (25 cm long;[22]). Stimuli consisted of two 5 cm^2 laminated black and white striped cards at 0.37 cycles per degree oriented horizontally or vertically. Both genotypes are capable of distinguishing this spatial frequency[26]. The position of the horizontal and vertical stimuli (left vs. right maze arm) was randomly altered across 30 trials with the constraint of equal number of trials in right and left arms. The 30 trial schedule changed each day, repeating every seven days. Random allocation determined which stimulus was rewarded in the learning phase, with the constraint that half the mice in each genotype received rewards for the horizontal and half for vertical. The rewarded stimulus was also counter-balanced across cage groups (i.e. mice housed together were rewarded for opposite stimuli) and sex. Inferential statistics confirmed no significant performance differences between sexes or stripe orientation first rewarded (Data File). Mice were placed at the start of the Y-maze, and received a peanut butter reward immediately after approaching the correct stimulus. If mice did not approach a stimulus after 30 seconds, the trial was deemed a non-response (included in analyses as an incorrect response). Each mouse completed 30 trials per day, in a single session. The reverse phase commenced the day after mice reached criterion performance (75% correct for three consecutive days). In the reverse phase the opposite, previously incorrect stimulus was rewarded. All other aspects of the reverse task were identical to the initial learning phase.

rTMS application

To deliver rTMS, we built a small coil created for mice (0.25 mm copper wire (Jaycar, Australia) 300 windings, 16 Ω, outer diameter 8 mm;[23]). The coil was designed to ensure a similar coil-to brain ratio as is used for induction of focal electric fields in humans[27] and was driven by an electromagnetic pulse generator (Global Energy Medicine, Australia). Under these conditions, the coil delivered a magnetic field of 10 mT. This relatively low intensity was imposed by the constraints of the coil's size but had the benefit of allowing us to evaluate the effects of stimulation without the confounding factors of stimulation-induced movement or the use of anaesthetic or restraint, with their associated changes to neuronal excitability and circulating stress hormones[28]. Furthermore, low intensity magnetic fields are clinically relevant for two reasons. Firstly, in humans, fields in the millitesla range delivered to the brain induce analgesia[29–31], and alleviate depression[32]. Secondly, even though traditional rTMS is considered to be focal, magnetic fields of lower intensity are delivered outside of the focal area[33], raising the possibility that low intensity stimulation may be contributing to therapeutic effects by acting on interconnected brain regions.

We chose a complex pattern of stimulation that is based on biomimetic principles (described in detail[23]; 59.9-ms trains of 20 pulses at 3 different frequencies as follows: 1 min warm-up at 6.71 Hz, 8 min treatment at 10.1 Hz, and 1 min cool down at 6.26 Hz) and has been shown to induce structural changes in mice[23]. The pulse was monophasic with a 300μs rise time and 100μs fall time. A Hall device probe (Jaycar, Australia) inserted into different brain regions of a euthanized mouse estimated that the dorsal hippocampus received roughly 6 mT when the coil was held 1 mm above the mouse's head, as described below. The surface temperature of the coil was measured after 10 min of stimulation and did not exceed 35°C.

As mice had completed the initial learning phase of the visual discrimination task before commencing rTMS they were accustomed to handling and remained relatively still without restraint. This allowed the stimulation coil to be held by the experimenter above the mouse's head. We thus delivered reproducible rTMS in the awake animal (as for cat studies[34,35]). Unlike in cat studies, the coil was not in direct contact with the mouse head but was held as close as possible to the scalp (~1mm). The gap between the coil and the head does not attenuate the field because magnetic fields decrease with distance from the source but are not modified by air or biological tissue (e.g. skin/scalp[36]). Unlike in the cat study, stereotaxic delivery was not attempted because the dimensions of the coil ensured that the field reached the entire dorsal hippocampus, which in the mouse, is relatively large in proportion to total brain size. Consistent with the low intensity of the magnetic field, mice did not display any head-eye or gross motor movements, nor altered behaviour in response to the stimulation. Sham stimulation involved the same procedure but with the stimulator switched off. This control was chosen as our coil did not produce any audible sound[23].

Golgi staining

Terminally anesthetised mice were transcardially perfused with 4% paraformaldehyde (Sigma Aldrich; Montana USA); Right hemispheres of brains underwent a silver impregnation staining protocol, and a Golgi stain (according to manufacturer's instructions: FD NeuroTechnologies, Maryland, USA), which allows the visualisation of morphology on a subset of neurons[37]. Briefly, hemispheres were incubated in the dark in solutions A+B for 8 days with a change into fresh solution after the first 24 hours. Hemispheres were then incubated in solution C for 4 days with a change into fresh solution after 2 days. The impregnated hemispheres were then cryosectioned at 100 μm on a Leica Cryostat CM1900 at -19°C, mounted onto glass slides (Thermo Fisher Scientific, Australia) subbed with 0.5% gelatin (Sigma Aldrich, Montana USA). Sections were dried in the dark for 2–7 days, washed in distilled water and developed in solution D+E for 10 minutes. Sections were dehydrated in increasing concentrations of ethanol and mounted in Entilin (Merck, Darmastadt Germany).

Imaging and analysis

Slides were analysed by a researcher blinded to stimulation condition and genotype. Sections were photographed by an Olympus DP70 digital camera at a 4× objective zoom, which encompassed the entire section. We analysed dendrites that could definitively be attributed to cells in the CA1 pyramidal and the molecular dentate layer of the hippocampus because dendritic spines on these cells have previously shown changes in dendritic spine density in

response to various interventions[38]. Dendrites were deemed suitable for analysis if they had a relatively flat orientation and were uniformly and strongly stained. Photographs of the dendritic arbour were taken throughout multiple planes ensuring the entire arbour was photographed in focus for later image analysis. Between 2 and 12 cells were analysed per animal and values averaged within animals for statistical analysis. Due to variability in Golgi staining, the number of dendrites counted varied between regions (CA1 pyramidal layer number of cells, Mean = 5; dentate molecular layer, Mean = 4.4).

The images for each dendritic arbour were combined into a single image, using the Image J plug-in "Stack Focuser" and dendrite length and number of dendritic spines counted using the Image J ("Cell counter" plug-in). The number of spines and the dendrite length data were then used to calculate a dendritic spine density value, defined as the number of spines per unit length (10 µm).

Statistical analysis

We examined the effect of long-term rTMS on reverse learning performance in ephrin-A2$^{-/-}$ and wildtype mice. Inferential statistics confirmed no pre-existing differences between groups in the learning phase, before commencing rTMS (data not shown). The first day of reverse phase training was excluded from analyses as rTMS commenced after this training session. Two mice reached the learning criterion in the reverse phase and were terminally anesthetised before 35 days (one wildtype and one ephrin-A2$^{-/-}$, both received sham stimulation (negative/handling controls)) thus there was non-random reduction in sample size over time, precluding use of daily performance measures in statistical analyses. To overcome this problem, data were divided into three blocks for each subject, with one third of total days training included in each block, reflecting early, middle, and late stages of the reverse learning phase. Mean percentage correct was analysed by a two-way mixed ANOVA to assess differences between stimulation conditions (rTMS vs. sham) and between genotypes (ephrin-A2$^{-/-}$ vs. wildtype) and changes over time (changes between early, middle and late blocks). As circuitry abnormalities and connections between measured hippocampal regions have not been characterised in ephrin-A2$^{-/-}$ mice, it is unknown whether these measures should be considered independent for statistical analyses. Accordingly, a MANOVA was conducted to assess effects of stimulation condition (rTMS vs. sham) and genotype (wildtype vs. ephrin-A2$^{-/-}$) on dendritic spine densities in both regions. Pillai's Trace (V) was used as the multivariate test statistic. Follow-up ANOVAs were conducted separately for each region, testing the same factors as used in the MANOVA. The F-test statistic (F) and probability (p) values are reported for each ANOVA. When the assumption of sphericity was violated degrees of freedom were adjusted with Greenhouse-Geisser correction. Data were analysed using SPSS statistics software (IBM Corporation, New York, USA, v.20).

Behavioural results (% accuracy) and average spine density for all mice

1 Data File

http://dx.doi.org/10.6084/m9.figshare.786497

Results

rTMS had no significant effect on reverse learning performance

As shown in Figure 1, groups had similar means in each block, with no significant difference between rTMS and sham, F (1, 16) = 0.28, p = 0.60, nor between genotypes, F (1, 16) = 0.86, p = 0.37, and no significant interactions across blocks (all p values >0.05). Within

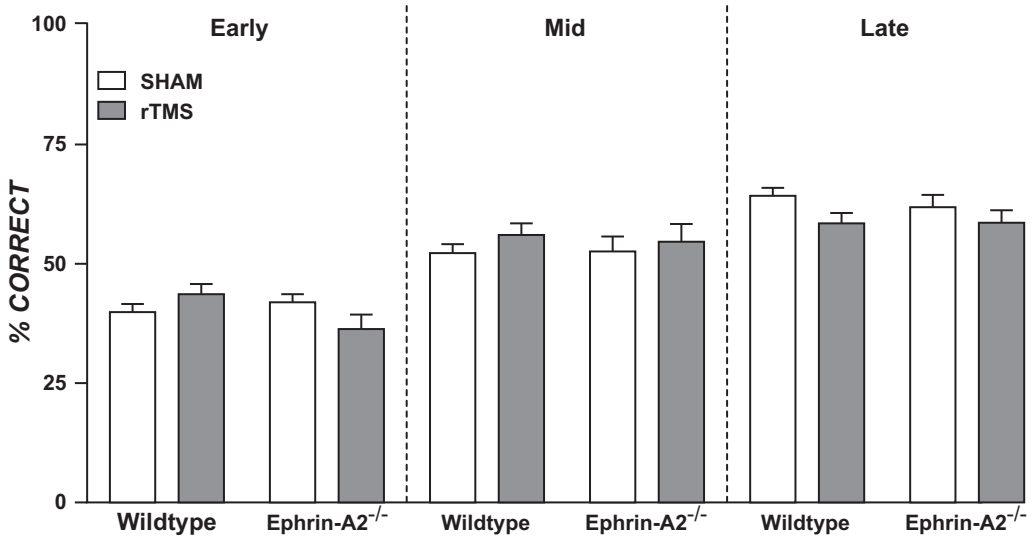

Figure 1. Mean percentage correct in a reverse-learning task for wildtype and ephrin-A2$^{-/-}$ mice receiving daily rTMS. Early, middle (mid) and late blocks were delineated by the first, second and final third of total number of days training in the reverse-learning task. Within groups, scores increased significantly between blocks (p <0.001), but there were no significant differences between rTMS and sham or between genotypes (p values >0.05; ANOVA). Error bars = SEM.

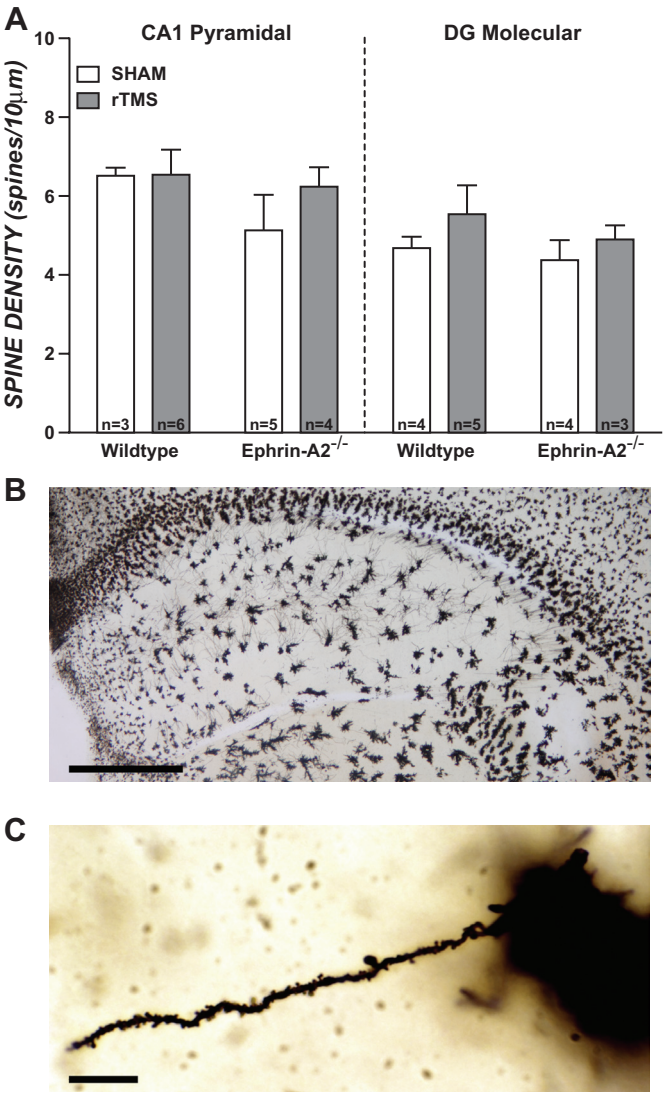

Figure 2. Assessment of rTMS effects on dendritic spine density in wildtype and ephrin-A2$^{-/-}$ knockout mice. (**A**) Mean dendritic spine density (number of spines per 10 μm) for hippocampal regions: CA1 pyramidal layer and dentate gyrus molecular layer (DG Molecular). There were no significant effects of rTMS nor genotype on spine densities in either region (p values >0.05; ANOVA). Error bars = SEM. (**B**) Right hemisphere Golgi stained section of dorsal hippocampus representative of those used in analyses. Scale bar represents 500 μm. (**C**) Dendrite representative of those selected for analysis, with spines visible. Scale bar represents 10 μm.

groups, the percentage correct increased significantly across blocks, indicating that mice adjusted to the rule reversal and successfully learned the task although this was not to the desired criterion (F (1.43, 22.87) = 71.80, p < 0.001 (Greenhouse-Geisser corrected)).

rTMS did not significantly affect hippocampal dendritic spine density

Spine density (spine number/10 μm) measures were obtained for each dendrite and averaged within mice. Figure 2 presents mean dendritic spine density in CA1 pyramidal layer and dentate molecular layer for ephrin-A2$^{-/-}$ and wildtype mice, contrasting rTMS to sham. There was a slight (non-significant) trend towards rTMS increasing spine density in ephrin-A2$^{-/-}$ mice in both regions. In wildtypes rTMS appeared to have no effect on spine densities in CA1 pyramidal cells, as means were almost identical. However, wildtypes showed a similar (non-significant) trend to ephrin-A2$^{-/-}$ mice in the dentate molecular layer.

MANOVA, using Pillai's trace showed there was no significant effect of stimulation condition, V = 0.13, F (2, 9) = 0.67, p = 0.54, nor genotype, V = 0.08, F (2, 9) = 0.39, p = 0.69, on spine densities. Follow up two-way ANOVAs were also performed separately for each region, with no significant differences between stimulation conditions or between genotypes in either region, all p values were >0.05 (CA1 pyramidal layer: stimulation condition, F (1, 10) = 0.03, p = 0.86; genotype, F (1, 10) = 0.86, p = 0.38. Dentate molecular layer: stimulation condition, F (1, 10) = 1.03, p = 0.34; genotype, F (1, 10) = 0.42, p = 0.53).

Discussion

We investigated the effects of long-term daily rTMS on learning and hippocampal dendritic spine density using ephrin-A2$^{-/-}$ mice and wildtype controls. We show that rTMS had no significant effect on learning and no significant effect on hippocampal dendritic spine densities. Although ephrin-A2$^{-/-}$ mice have abnormal brain circuitry and associated abnormal behaviours, in the present study, the previously reported learning deficit[18] was not observed due to low levels of food deprivation[22]. Although it is difficult to draw conclusions from the null results presented here, the absence of observed behavioural and structural change is consistent with previously reported rTMS specificity for abnormal systems[8,23]. The lack of adverse effects in our long-term study suggests that up to 5 weeks of daily sessions of low intensity pulsed magnetic field stimulation at the parameters used in this study appears safe to use in healthy participants.

Long-term rTMS does not adversely affect learning in normal subjects

We originally hypothesized that rTMS would rescue the learning strategy deficit in ephrin-A2$^{-/-}$ mice[18] with minimal or no effect on wildtype mice. However, both genotypes failed to demonstrate the strategy deficit, due to insufficient food restriction[22]. Our results nonetheless indicate that rTMS does not adversely affect performance, but nor does it improve motivation or accelerate learning when deficits are absent. This is consistent with previous reports that long-term rTMS effects are specific to abnormal brain circuitry: two weeks of rTMS improved visual tracking, visual electrophysiological function and topographical accuracy in a different strain of mice (ephrin-A2A5$^{-/-}$ double knockouts) with abnormal circuitry but produced no lasting effects in wildtype mice[23]. Human studies also support specificity of rTMS for abnormal brain circuits, as a meta-analysis of rTMS effects on cognitive performance found patients tend to improve more than healthy participants[8]. Although some human studies using healthy participants show a single-session of rTMS enhances cognitive task performance, such as analogous reasoning[39] and reaction time[7], results are mixed, with other studies showing no effect of rTMS on knowledge acquisition[40] or accuracy in a go/no-go task[41]. Furthermore, there is a lack of studies assessing cognitive effects of long-term rTMS in patients together with healthy controls, which presents a large gap in knowledge[8].

Because of the lack of understanding of fundamental interactions between rTMS and behaviour, it would be of great interest to perform an exhaustive battery of behavioural tests in healthy wildtype mice (and eventually in animal models of disease) in conjunction with various rTMS protocols. Subsequent anatomical and physiological analyses could then be carried out to elucidate the neural mechanisms of rTMS and gain insight into the treatment of human disease.

Long-term rTMS and hippocampal spine density

Each mouse received a controlled amount of daily rTMS, allowing us to investigate how long-term rTMS combined with daily training influences spine density. Importantly, we found similar spine densities in sham wildtype and ephrin-A2$^{-/-}$ mice, suggesting that spine density is not solely dependent on ephrin-A2, in agreement with the literature[19–21]. As such, the null effect of rTMS on dendritic spine density may be attributed to the absence of both a specific spine and learning deficit in both wildtype and ephrin-A2$^{-/-}$ mice. It will be important to examine other brain regions to determine whether the selectivity of rTMS for normal and abnormal brain circuits is also observed. Our result that spine density was not significantly altered is in agreement with a previous study, showing no change in spine density in CA1 pyramidal neurons following a single rTMS stimulation[2]. However, it is surprising that dendritic spine density remains unaffected after long-term stimulation, given our previous results using the same stimulation parameters, demonstrating structural reorganisation in abnormal axon terminals following multiple stimulations, but not a single rTMS session[23]. As neither long-term nor short-term rTMS results in dendritic spine density changes, these negative results may suggest different susceptibility of axons and dendrites to rTMS. The functional characteristics of these neuronal compartments require different expression of ion channels and growth factor receptors[42], which could provide a molecular basis for differential rTMS effects on excitability and spatially localised structural and functional change.

Alternatively, the timing of rTMS delivery relative to the behavioural task may have influenced the outcome of our experiments. Here we stimulated after the task, however rTMS might have been more effective if delivered before. Because a single session of rTMS increases the size of dendritic spines and may activate silent synapses[2], this may "prime" the brain for learning. With such pre-treatment, an effect of rTMS might even have been detected in improved performances on a day to day basis.

An alternative interpretation of our null finding is that our rTMS treatment changes spine dynamics without affecting their final density, a result that would not be possible to detect in our fixed post-mortem tissue. Hence, these results highlight the limitations in Golgi staining of fixed tissue, a technique still commonly used in examining dendritic spine density. Sensory manipulation (either enrichment or withdrawal) strongly alters spine dynamics *in vivo* in various areas of the cortex of adult mice[43]. As a rule, established spines are pruned during the initial experience of the new stimulus, while new ones are established, which may result in some studies of fixed tissue showing no net change in spine density[44,45]. Consistent with the change in spine dynamics initiated by enrichment, a recent imaging study in the hippocampus identified two phases in spine dynamics following repeated induction of LTP. Initially both generation and retraction of spines increased, followed by a cessation of spine retraction[46]. This is consistent with post-mortem studies showing an initial period of apparent spine stability, followed by a detectable increase in density. The possibility that rTMS changes spine dynamics, as opposed to density, is further supported by an increase in the size of small spines following a single stimulation, which the authors suggested may indicate the activation of silent synapses by membrane recruitment of AMPA receptors, precluding the need for de novo synapse generation[2]. Future live imaging studies of spine dynamics in animals that have received single or multiple rTMS stimulation, potentially in combination with learning tasks will provide much needed insight into the mechanisms underpinning the plastic changes elicited by rTMS in humans.

Importantly, we are conscious of the limitations of our rodent scaled rTMS delivery device which may have contributed to the lack of effect observed here. Although our coil had a relevant coil to brain ratio for mice, because of its small size, the intensity of the magnetic field did not reach the magnitude commonly used in humans (6mT compared to 1-2T), raising concern that our stimulation paradigm is not comparable to human rTMS. However, this raises a more general issue because similar criticism applies to studies that employ larger coils[1–3]: although these deliver the same fields used in humans, the focal nature of the stimulation is lost. Additional effort in designing appropriate small animal rTMS coils is urgently needed to improve the construct validity of animal rTMS research.

Author contributions

JR conceived the study, JR and KM designed the experiments, MS and KM carried out the research and analysed results. JR and KM wrote the manuscript and all authors were involved in the revision of the manuscript and have agreed to the final content.

Competing interests

No relevant competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Current Referee Status:

Referee Responses for Version 1

Anthony Hannan
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Approved: 21 October 2013

Referee Report: 21 October 2013
This is a well written paper describing the effects (or lack thereof) of rTMS over 5 weeks in wild-type and ephrin-a2 knockout (KO) mice. The data are clearly presented in a usable format and are discussed appropriately. The initial referee Antoni Valero-Cabre, who is an expert in TMS and neuro-stimulation, has already extensively covered the key points, both positive and negative. I am therefore only going to make a few additional comments.

The title and abstract should ideally reflect the findings of the study more precisely, for example:

'Long-term delivery of pulsed magnetic fields does not alter visual discrimination learning or dendritic spine density in mouse CA1 pyramidal or dentate gyrus neurons'.

The nature of such negative findings means that the authors cannot rule out potential significant effects of this rTMS protocol on other learning tasks or other classes of neurons/dendrites in the mouse hippocampus. Furthermore, the abstract should mention that they were adult mice and the background strain used was C57Bl/6J, as it is possible that different aged mice and/or a different genetic strain of mice might respond differently to the same rTMS protocol.

One concern regarding the methods and design is that the authors appear to have used a separate wild-type mouse colony and compared them to ephrin-a2 KO mice inbred via a backcrossed colony. I strongly feel that all such experiments should always involve wild-type littermates randomised from age-matched litters to control for both genetic (e.g. sub-strain and genetic drift) and epigenetic differences between different colonies. This is particularly important for behavioural experiments where even subtle genetic and epigenetic differences can often have significant impacts. The authors report no significant gene effects, and it is therefore not a major confound in this case, however if randomised littermates were not used (as the methods imply) this should be noted.

The discussion and conclusions are balanced. However, it might be worth noting that an extensive battery of behavioural tests (for example including sensory, cognitive, affective and motor protocols) on wild-type mice would be worth pursuing to assess whether this rTMS protocol (or others with different spatial and temporal specificities) has any effects on brain function analogous to human studies. If any positive results were found, then cellular, physiological and molecular follow-up studies could be targeted towards understand specific cognitive/behavioural effects of rTMS (thus nicely complementing human studies).

If such comprehensive TMS animal studies are done but still show inconsistencies with matching human TMS studies then one possibility is that the animal TMS needs to better match the exact spatial, temporal and biophysical aspects of human TMS. For example, the human brain is much larger, the skull much

thicker and the spatial extent and physical effects of a given TMS coil are no doubt difficult to match. The need to further improve such 'construct validity' for animal TMS may also be worthy of further discussion.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Antoni Valero-Cabre
School of Medicine, Anatomy and Neurobiology, Boston University, Boston, MA, USA

Approved: 26 September 2013

Referee Report: 26 September 2013
This is an outstandingly planned and well-executed study that shows that the rodent brain, after a daily 10 minute exposure to low field pulsed magnetic stimulation for 5 weeks (i.e. 35 sessions) exhibits null effects on learning behavior and hippocampus dendritic spine density in both Wild Type and Ephrine A2-/- mice. The effort of the authors deserves a lot of credit, as studies like this one are extremely hard, long and laborious to perform and the possibility of significant results always uncertain. In spite of the current null results, this kind of work is greatly needed in the field of non-invasive brain stimulation in order to understand the cellular underpinnings of these techniques and assess the safeness and real therapeutic potential of stimulation technologies in clinical neurorehabilitation. Overall, the neurostimulation community should strongly thank the authors for investing their know-how, time and effort to carry over this type of animal research and we all should encourage them to pursue their efforts in the future.

GENERAL POINTS

The only limitation of the current manuscript is related to the difficulties in interpreting null outcomes; in this case a triple negative result. Not only did low field pulsed magnetic stimulation prove unable to modify visual learning behavior or hippocampus spine density in these two population of mice but neither of the two showed (as should have been at least expected) significant differences in any of these two measures. The authors do an excellent job going over the different possibilities that could explain this null pattern of outcomes, even if they do not have much basis to rule out any of the hypotheses in particular. Among other important issues, the study emphasizes once more the importance of task titration. Task titration is strongly related to animal motivation (and thus intake restriction) and is a key variable and therefore needs to be manipulated carefully to make sure that the potentially effective impact of magnetic pulses can be demonstrated behaviorally. With regards to the question however of whether or not increasing food restriction could have rendered, as the authors suggest, behavioral learning tests more sensitive, the answer remains unknown. It could also be that highly motivated animals would rapidly show ceiling learning effects and render the task less sensitive to modulation. To this reviewer, the performance of both groups of animals in the inversion phase of the task seems to show enough room for behavioral improvement, even after 5 weeks of stimulation; thus it is tempting to also speculate that such a complex task for a rodent (which relies on several cognitive processes such as visuo-spatial attention, visual perception, visual, spatial and declarative memory, associative rewarded learning, rule understanding and switching and decision making) may not have been well suited to the functions of the brain regions aimed at in this study and/or that their impact could have rapidly compensated for by other systems or regions.

Adding to the authors' arguments with regards to the lack of spine density effects, one could also argue

that a direct impact of the stimulation (and not an indirect effect of the stimulation on learning behavior driving subsequent effects on memory related regions) on such measures could and should have occurred primarily in the cortical regions overlying the hippocampus (which have not been analysed in this study) rather than in the more deeply located hippocampus CA1 and DG neurons. Of course, if the targeted region theoretically related to the behavior that is being measured is not at least directly or indirectly impacted and modified in activity by the pulses, then no behavioral differences between sham and real rTMS patterns should be expected in either population of mice. All these explanations are directly or indirectly mentioned in the discussion and when not explicitly mentioned in the text, they emerge logically from the results. However any effort to nuance and expand such justifications even further would be welcome.

To this reviewer, the only possibility (that would also be compatible with the negative outcomes of this study) that is not explicitly mentioned in the discussion is that the delivered low field pulsed magnetic patterns (even if estimated by means of a Hall device in postmortem brains as reaching intensities of ~6 mT in the hippocampus) were not effective at all. Several reasons that could account for that eventuality are; that the field was too weak to penetrate deep enough with a minimal intensity, imprecise widely distributed targeting or a field strongly attenuated by the space left, as indicated in the manuscript, between the stimulating coil and the top of the head. This reviewer cannot demonstrate that this possibility is more likely than any of the others already mentioned in the manuscript. Nonetheless, in the absence of any positive sign of a stimulatory impact, this possibility cannot be ruled out and should probably be briefly discussed. Additionally, it should be clear throughout the manuscript, that the ability of the current experiment to highlight the innocuity of 5 weeks of low field pulsed magnetic brain stimulation needs to be interpreted carefully and associated with the field strengths delivered by the modified e-cell device by Global Energy Medicine, which is far from the normal field strength for TMS/rTMS devices operated in humans for either research or therapeutic purposes.

MINOR COMMENTS AND SUGGESTIONS

1. *Introduction, Page 1, Col 1, Par 1, Line 6.* Could the authors re-evaluate the adequacy of the term “metaplasticity” with regards to TMS-induced LTP effects, as to this reviewer, the main effect of high frequency stimulation are LTP or LTP-like effects. Metaplasticity is a consequence of forcing homeostatic plasticity beyond a particular boundary, which may result in paradoxical modulatory effects.

2. *Introduction, Page 1, Col 1, Par 2, Lines 7-9*. With regards to this issue, in this particular study were the authors hypothesizing a direct impact of low field pulsed magnetic stimulation on specific cortical locations translated through connectivity into a declarative and spatial memory region such as the hippocampus, or a cortical effect of stimulation leading to subsequent changes in behavior that could leave a distinctive memory trace in the hippocampus?

3. *Introduction, Page 1, Col 1, Par 3*. The paper by *May et al. 2007, Cereb. Cortex,* in which the effect of 5 straight days of rTMS on humans assessed with MRI methods and regional size increases (hypothetically attributed to increases in spine density) could be relevant for this introduction.

4. *Methods, Page 1, Col 2, Par 1, Lines 7-8.* Could the authors provide an idea of how far in their development (if possible in age compared to humans) are 8-10 week old mice? It seems that in a prior study by the same authors that showed significant results after 14 days of low field rTMS on the visual cortex by *Rodger et al. 2012, FASEB J,* the mice were slightly younger (6-8 weeks). Is this period crucial in terms of postnatal development for mice? Could a less plastically sensitive

brain of 8-10 week old mice vs. 6-8 week old mice explain the current null results? Please comment briefly in the manuscript.

5. *Methods, Page 2, Col 2, Par 2, Lines 6-7.* Why were mice tested before the low field pulsed magnetic stimulation session and not also immediately thereafter, when the impact of the stimulation should have been stronger? Although I understand the authors sought a long-term effect, such a measure could have proven useful to reveal at least an immediate day-to-day impact of stimulation and become a proof of their efficacy? Given the order of events, one could be tempted to speculate the possibility of non-synergistic interaction between the lasting effects of task practice and immediately subsequent low field rTMS stimulation, cancelling the modulatory effects.

6. *Methods, Page 3, Col 1, Par 2, Lines 7-8*. As this is not a standard rTMS stimulation device, some additional information on the stimulation source should be given to be able to compare the efficiency of low field pulsed magnetic stimulation with current human rTMS equipment. More specifically, what is the shape of the stimulating pulse (monophasic, biphasic?) and what is the rise time of the field? The pattern of stimulation used seems essentially an “excitatory” 10 Hz rTMS pattern, preceded and followed by short instances of ~6 Hz stimulation. Was this high frequency employed as a way to induce LTP phenomena in the hippocampus or as a tool to enhance cortical excitability and facilitate learning behavior leading then to functional and anatomical modulations of the hippocampus? Please comment briefly.

7. *Methods, Page 3, Col 1, Par 2, Lines 7-8.* Navigation of the TMS coil of course could have been improved given the high-focality of the device used by the authors and the fact that any small head movements in a small rodent brain could easily lead to a completely different area of stimulation. It is true that prior studies in awake felines by our own group (see *Valero-Cabré et al. 2006, Exp Brain*, *Valero-Cabre A et al. 2008, Eur J Neurosci.*) have used a similar manual procedure, but it is also the case that the coils and brains in those studies were larger and precise location on a specific region of the posterior parietal cortex was guided day to day by stereotaxic based measures and references. In many instances, we additionally benefited from parietal bone transparency during surgery, and identified in each animal the sulci/gyral pattern and labelled the exact area of interest (see *Valero-Cabré et al. 2005 Exp Brain Res, Valero-Cabré et al.2007 Exp Brain Res*). Furthermore, in groups that were to be followed longitudinally, we placed a dental acrylic plug, that could be palpated through the skin of the scalp (see *Afifi et al. 2012 Eur J Neurosci*) which was then used as consistent localization marker. The correct positioning of those plugs with regards to the stimulated areas was often verified pre-treatment by anatomical MRI methods and also in post-mortem brain dissections at the end of the follow up. In the current study, given the elongated shape of the hippocampus in rodents, which part of the rodent cortex or which stereotaxic level was aimed at during the stimulation? Also, in contrast with the quoted feline study, the authors indicate here that the coil was not in direct contact with the scalp. As air is a strong isolator, could that have attenuated the strength of such a low intensity field even further? Were Hall probe field measures in the hippocampus performed with the stimulating cell also separated from the scalp?

8. *Discussion, Page 5, Col 1, Par 1, Lines 4-6.* The statement “*The absence of observed behavioral and structural change is consistent with previously reported rTMS specificity for abnormal systems*” needs to be expanded upon as the Ephrin-A2-/- mice have indeed abnormal systems. Maybe a short comment on how to reconcile the current findings with those reported in *Rodger et al. 2012, FASEB J*, would be important as in that study by the same authors similar stimulation patterns

were able to correct cortico-collicular connectivity only in Ephrin-A2-/- and not in Wild type mice, supporting the above-mentioned statement.

9. *Discussion, Page 5, Col 1, Par 1, Lines 6-8*. I would strongly advise the authors to alter the contents of the sentence "*Furthermore, the lack of adverse effects in our long term study contributes evidence that rTMS is safe to use in healthy control participants*". Could they rephrase to something like "Furthermore, the lack of adverse effects in our long term study suggests that up to 5 weeks of daily sessions (35 session) of low field pulsed magnetic stimulation at the parameters used in this study appears safe to use in healthy participants". This is important as the intensity levels employed are very different of those employed in human rTMS, which are much higher, and this information could have some public health implications for future use of rTMS in humans.

10. *Discussion, Page 5, Col 2, Par 1, Lines 19-21*. Please check the accuracy of the sentence "*To our knowledge, there have been no studies assessing cognitive effects of long-term rTMS in patients and healthy controls, which presents a large gap in knowledge*"

11. *Discussion, Page 5, Col 2, Par 1, Lines 21-22*. As indicated above (comment 10), would the authors agree to rewrite the sentence "*Although more research is needed, our results support that rTMS is safe to use in healthy control participants…*" and revise the second part of it "*… invaluable in assessment of rTMS effects clinically*" which to this reviewer is confusing?

12. *Discussion, Page 5, Col 2, Par 2, Lines 5-8.* This sentence can be a bit surprising to readers, why did the authors chose a knock out for Ephrine A2 in a study that aimed to analyze spine density in the hippocampus if as they affirm in this paragraph, this molecule is not involved in spine dynamics? Maybe the rational for that choice needs to be explained more carefully.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.